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ScienceDirect

Virology 361 (2007) 402–411

VIROLOGY

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Immunological changes and cytokine gene expression during primary infection with human T-cell leukaemia virus type 1 in squirrel monkeys (*Saimiri sciureus*)

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Received 18 September 2006; returned to author for revision 6 October 2006; accepted 17 November 2006
Available online 16 January 2007

Abstract

We have developed an animal model of experimental HTLV-1 infection in *Saimiri sciureus* monkeys in order to study both the immunological and the virological aspects of the infection. As cytokines expressed by immune cells play an essential role during viral infection, we have studied the correlation between the expression of some Th1/Th2 cytokines, including IL-2, IL-5, IL-6, IL-10, and IFN-γ, and immunological dynamics during primary and chronic HTLV-1 infection in this model. We first demonstrated that, during primary infection, IFN-γ, IL-2 and IL-10 are expressed at different times and levels. The expression of these cytokines is concomitant with the increase in the numbers of $CD4^+$, $CD8^+$ and $CD16^+$ cells and with the presence of *tax/rex* viral mRNA. These data indicate the involvement of various cell types in the antiviral immune response. Subsequently, we showed that peripheral blood mononuclear cells freshly isolated from chronically infected monkeys express IFN-γ and IL-6 at higher levels than those from uninfected animals. IFN-γ expression is quantitatively correlated to the proviral load and to the presence of circulating effector T-cells against Tax peptide, as detected by Elispot. Further studies will be needed to determine the effective role of these cytokines and other immune system modulators in the control of viral replication during primary HTLV-1 infection or latency.

Keywords: HTLV-1; Cytokine; Gene expression; RT-PCR; Proviral load; Cellular immunity

Introduction

Human T-cell lymphotropic virus type 1 (HTLV-1), the causative agent of adult T-cell leukaemia/lymphoma (ATLL) (Yoshida, 1983) and of tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM) (Gessain et al., 1985), has also been associated with paediatric infectious dermatitis (Lagrenade et al., 1990), uveitis (Mochizuki et al., 1992), arthropathy (Ijichi et al., 1990) and polymyositis (Morgan et al., 1989). Owing to the inherent difficulty of obtaining human specimens shortly after infection, a relevant animal model was essential for better understanding of host–virus interactions, virus transmission, the natural history of infection and the pathogenesis of HTLV-1-associated diseases. Such models could also be critical for testing candidate vaccines (Kazanji et al., 2001).

We have previously shown that the squirrel monkey, *Saimiri sciureus*, a South American non-human primate that is free of simian T-cell leukaemia virus, is susceptible to experimental infection with syngeneic or allogeneic HTLV-1-immortalized cells (Kazanji et al., 1997b). As in humans, such experimental inoculation leads to chronic infection, and the HTLV-1 provirus is still detectable by polymerase chain reaction (PCR) in the

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doi:10.1016/j.virol.2006.11.028

peripheral blood mononuclear cells (PBMCs) of these animals up to 7 years after inoculation.

We have also shown that PBMCs, spleen and lymph nodes represent the major reservoirs for HTLV-1 during the early phase of infection. In a previous study, in which we investigated the virological changes during primary infection, we showed that the HTLV-1 provirus could be detected by PCR as early as 6 days after infection. Competitive PCR showed that the proviral load increased in the PBMCs of infected animals shortly after infection and remained stable thereafter. RT-PCR and *in situ* hybridization indicated, however, that HTLV-1 *tax/rex* mRNA is transcribed only transiently during the early stage of infection (Kazanji et al., 2000).

The humoral and cellular immune responses to HTLV-1 were also evaluated at various times after HTLV-1 inoculation. Anti-HTLV-1 antibodies were not detected 6 and 12 days after injection; however, by 35 days, high antibody titres were present. Anti-p40*tax* and anti-*env* cytotoxic activity were also detected 2 months after infection and remained detectable thereafter (Kazanji, 2000; Kazanji et al., 2000). This CTL response in monkeys appears to be comparable to that observed in asymptomatic human HTLV-1 carriers. In carriers and in TSP/HAM patients, the CTL response is also directed mainly against the p40 Tax protein (Jacobson, 2002). It has been suggested that this cellular response plays a major role in controlling HTLV-1 replication and then in maintaining a low viral load (Bangham, 2000; Bangham, 2003; Bangham et al., 1996; Jacobson, 2002). In simian immunodeficiency virus infection, the prognosis of subsequent disease is likely to depend on the rate of viral replication relative to the antiviral immune response, especially during the first few days after infection. Furthermore, different cytokine profiles might influence the outcome. As there are no data on the dynamics of the cellular immune response during primary HTLV-1 infection, we decided to use our non-human primate model of HTLV-1 infection to study the evolution and role of this response in HTLV-1 infection. We previously developed a quantitative method based on competitive RT-PCR to evaluate the expression of various Th1 and Th2 cytokines, including IL-1β, IL-2, IL-5, IL-6, IL-10, TNF-α and IFN-γ, from squirrel monkeys in vivo (fresh PMBCs) (Heraud et al., 2002). In the present study, we evaluated the relation between the $CD4^+$, $CD8^+$ and $CD16^+$ cell response and the expression of some Th1 and Th2 cytokines, including IL-2, IL-5, IL-6, IL-10 and IFN-γ, during primary infection and during latency in squirrel monkeys. At a later stage of infection, the expression of these cytokines was compared with the proviral load and with the presence of circulating effector T-cells against Tax obtained from chronically infected animals.

Results

A transitory concomitant increase in T-cell response during primary infection

To investigate and identify the T-cell response, we first generated a polyclonal serum directed against squirrel monkey $CD8^+$ T-cells (see Materials and methods and Fig. 1), as none of the commercially available anti-$CD8^+$ preparations cross-reacted with the squirrel monkeys $CD8^+$ cell subset.

Two monkeys were inoculated with HTLV-1-transformed cells as previously described (Kazanji et al., 1997b), and lymphocyte and leukocyte counts and the anti-HTLV-1 humoral response were monitored for up to 3 months. As shown in Fig. 2, both animals showed a transient increase in lymphocytes and leukocytes, with a peak on day 7 after infection. The cell numbers decreased after 12 days and remained stable thereafter. HTLV-1 ELISA revealed a positive antibody response against the virus 30 days after inoculation (Fig. 2), which remained at a high level thereafter. To confirm these observations, and to evaluate whether the increase in the white blood cells and lymphocytes count was or not due to an allogeneic response to the injected cells, five additional animals were inoculated. Four monkeys (A050C, A115C, 96070 and A029C) received the HTLV-1-transformed cell line as described above and one control monkey (A118C) was given the same number of Jurkat cells. As seen in Fig. 3, all four HTLV-1-infected animals showed increased absolute numbers of lymphocytes as well as in WBC (data not shown). In particular, we observed increased numbers of both $CD4^+$ and $CD8^+$ T cells between days 4 and 14, with a peak between days 7 and 9 (Fig. 3). Two of the four animals (A050C and A115C) also showed a significant increase in the number of $CD16^+$ cells, with a peak at day 7. The cells of the control animal showed no significant modification.

Expansion of T cells and natural killer (NK) cell subsets during viral infection might be concomitant with the expression of various cytokines involved in the immune response. To assess this idea, we measured the mRNA expression of IL-2, IL-5, IL-6, IL-10 and IFN-γ, which are known to play an important role in the humoral and cellular response during primary infection.

IFN-γ, IL-2 and IL-10 expression and detection of HTLV-1 tax/rex mRNA during primary HTLV-1 infection

IL-2, IL-5, IL-6, IL-10 and IFN-γ expression was evaluated by RT-PCR up to 2 weeks after infection in the four infected animals and in the control animal. The animals did not differ significantly in the expression of IL-5 and IL-6 (data not shown); however, IL-10 and IFN-γ expression was observed on days 4–14 in the infected animals but not in the control (Fig. 4). Transitory expression of IL-2 was observed from day 4 to day 7. Infected monkeys showed peak expression of IL-10 on day 7, which decreased significantly during the next few days.

To determine the relation between these observations and HTLV-1 expression during the same period, the expression of *tax/rex* mRNA was investigated by RT-PCR. *Tax/rex* mRNA was detected only from day 7 to day 11 but was undetectable by day 14 (Fig. 4).

Cytokine expression in chronically HTLV-1-infected monkeys and association with HTLV-1 proviral load

To investigate the role of these cytokines at a later stage of HTLV-1 infection, we also evaluated their expression in fresh

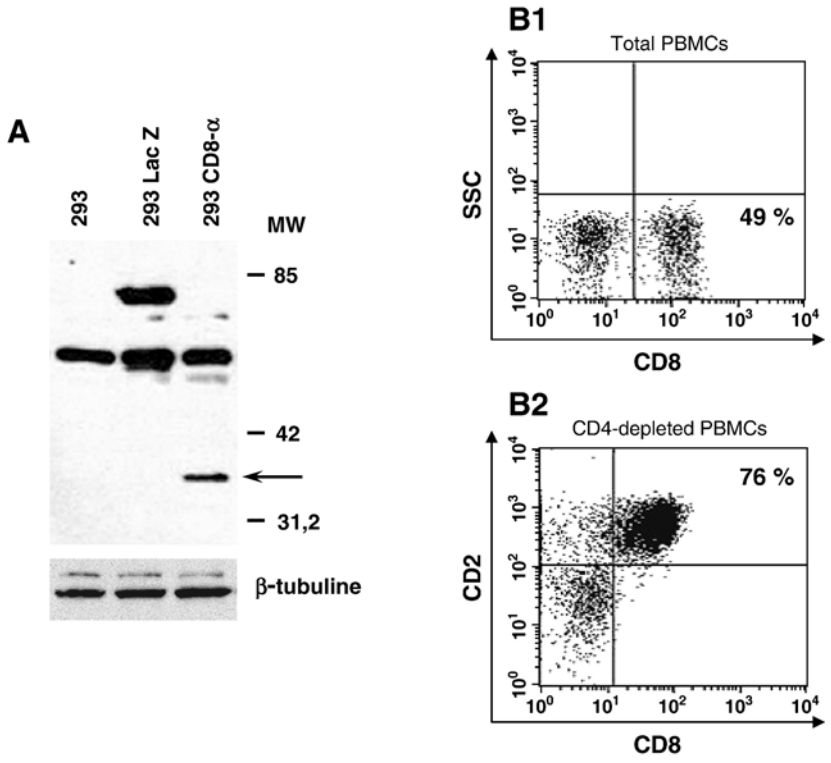

Fig. 1. Detection of *Saimiri sciureus* CD8 α-chain expression and flow cytometry analysis of $CD8^+$ T cells labelled with anti-squirrel monkeys CD8 cells. (A) Lane 1, mock-transfected 293T; lane 2, 293T cells transfected with plasmid pcDNA4/HisMax-Lac Z; lane 3, 293T cells transfected with recombinant pcDNA4/HisMax-CD8-α plasmid. Antibody against β-tubulin was used as control for protein loading. (B1 and B2) Newly generated polyclonal directed against squirrel monkey (*S. sciureus*) CD8 α-chain can detect cells expressing this cell marker. Cytometric analysis revealed that 49% of total PBMCs were $CD8^+$ cells (B1), and 76% of $CD2^+$ cells from $CD4^+$-depleted cells fraction were $CD8^+$ cells (B2).

PBMCs obtained from four other chronically HTLV-1-infected monkeys. We found no difference in IL-2, IL-5 and IL-10 levels as compared with uninfected monkeys (Fig. 5) and, higher IL-6 and IFN-γ expression was found in most HTLV-1-infected animals, as the expression of these two cytokines is very low or undetectable in uninfected animals.

To determine which T-cell types express IFN-γ, $CD4^+$ and $CD8^+$ cells were purified, and IFN-γ expression was evaluated

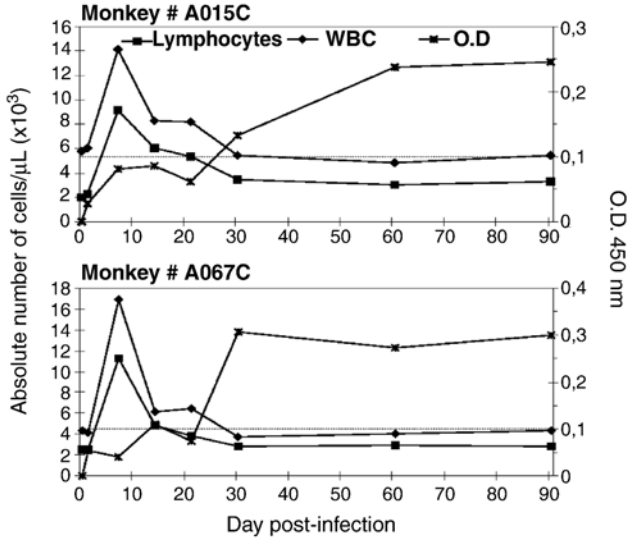

Fig. 2. Immunological response during primary HTLV-1 infection in squirrel monkeys. Blood samples were collected on days 0, 1, 7, 14, 21, 30, 60 and 90 after inoculation, and leukocytes and lymphocytes (WBC) were counted. During the same period, the antibody response against HTLV-1 was measured by ELISA (the dotted line represents the cut-off value). OD, optical density.

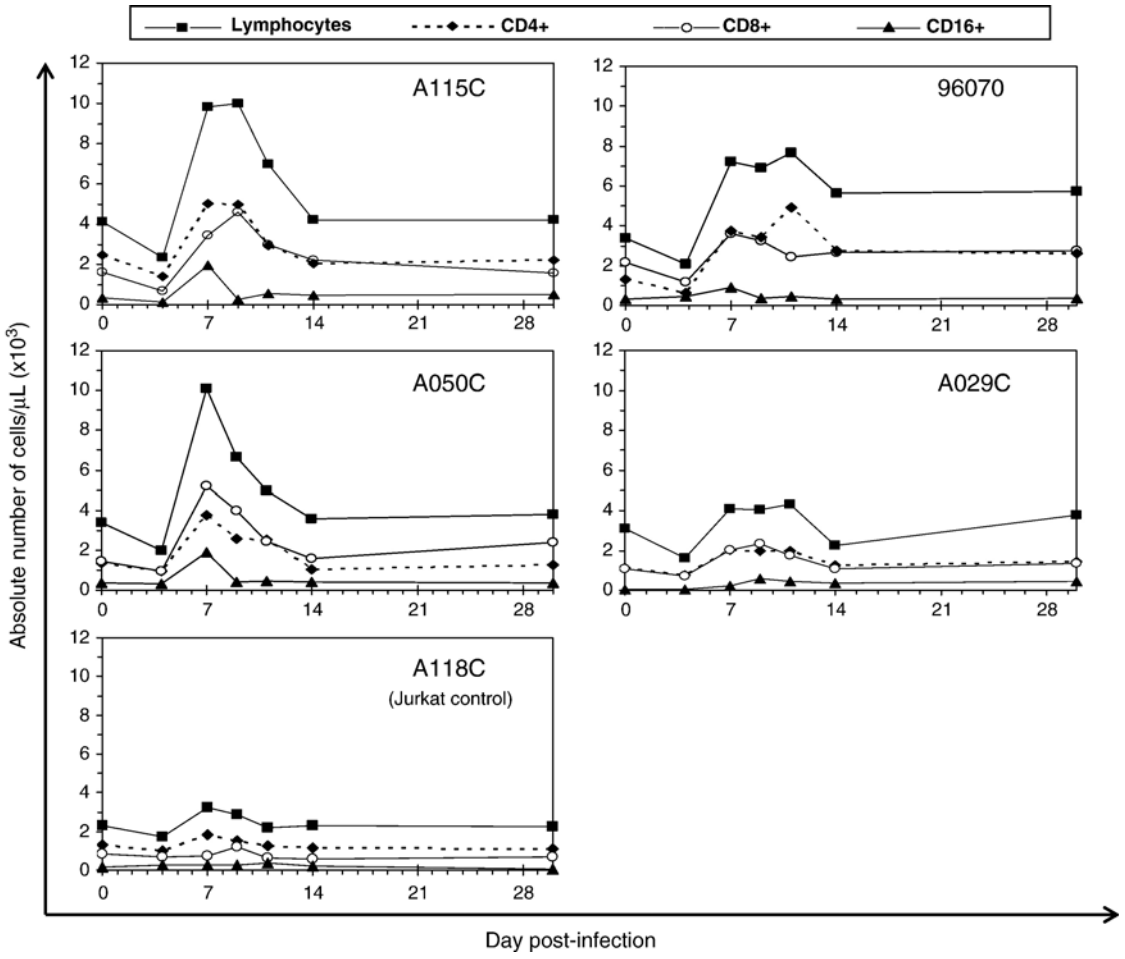

Fig. 3. Absolute numbers of peripheral $CD4^+$, $CD8^+$and $CD16^+$ cells during primary HTLV-1 infection in squirrel monkeys. Four animals (A115C, 96070, A050C and A029C) were inoculated with an HTLV-1-transformed cell line (EVO/1540). On days 0, 4, 7, 9, 11, 14 and 30 after infection, lymphocytes and $CD4^+$, $CD8^+$ and $CD16^+$ cells were counted by cytometry. As a control, one animal (A118C) was inoculated with Jurkat cells.

as described above. As seen in Table 1, both $CD4^+$ and $CD8^+$ T-cells expressed IFN-γ. $CD4^+$ T cells were the main IFN-γ-expressing subset in two HTLV-1-infected animals (1491 and 94119), whereas $CD8^+$ was the main subset in animal 1540. There was no difference between $CD4^+$ and $CD8^+$ T-cell IFN-γ expression in the fourth monkey (1715) (Table 1). We further evaluated the relation between IFN-γ expression and HTLV-1 proviral load. Statistical analyses of the data presented in Table 1 using the Spearman's Rank Correlation Test demonstrated a strong positive correlation between the expression of IFN-γ in total PBMCs and the proviral load (Spearman rank correlation coefficient, 0.97 with $p=0.033$). Nevertheless the same test performed on $CD4^+$ and $CD8^+$ T-cells subset revealed only a positive but non-significant relation between IFN-γ expression and proviral load ($r_s=0.82$, with $p=0.13$, for $CD4^+$ cells, and $r_s=0.40$, with $p=0.10$, for $CD8^+$ cells).

Frequency of circulating effector T-cells against Tax in chronically HTLV-1 infected monkeys and relation to the proviral load

The frequency of circulating effector T-cells against Tax was evaluated by measuring IFN-γ induction by Elispot in fresh PBMCs obtained from the four chronically HTLV-1-infected monkeys. We used 32 overlapping peptides covering the entire Tax protein (Parker et al., 1992), collected in 11 pools (each pool containing three overlapping peptides of 9–10 amino acids) (Table 2). As seen in Fig. 6, stimulation of PBMCs with Tax antigen induced a higher frequency of IFN-γ-secreting cells in two HTLV-1 infected monkeys (1715 and 1491) than in the other ones (1540 and 94119). None of the 11 pools induced a significant response in two uninfected control animals (data not shown). As shown in Table 1, monkeys 1715 and 1491, which had higher frequencies of circulating effector T cells, also had the highest proviral loads.

Discussion

In all newly infected animals, an increased level of IFN-γ expression was observed during primary HTLV-1 infection, concomitantly with an increase of the numbers of $CD4^+$, $CD8^+$ and $CD16^+$ cells. The expression of this cytokine at an early stage of HTLV-1 infection is probably the result of T cell expansion as a mechanism to control the infection and viral spread. Interferons are well known for their antiviral properties, their regulation of cell growth and their immunomodulatory

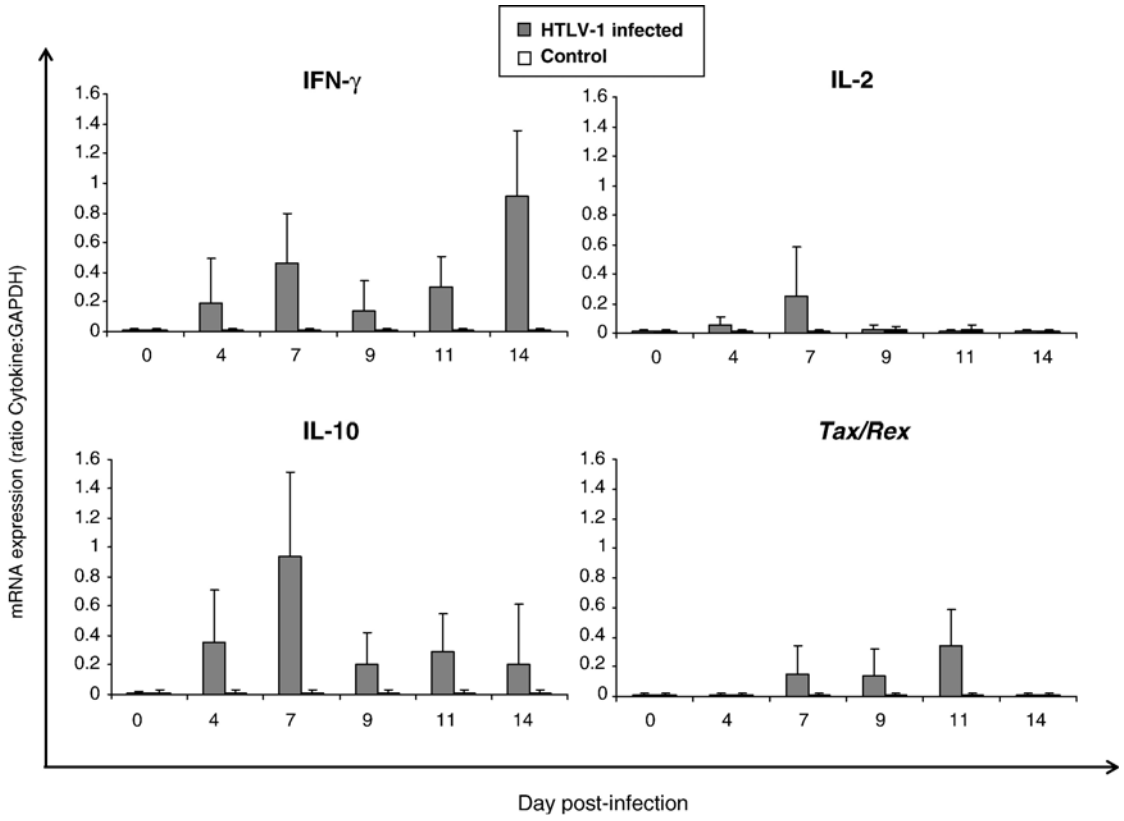

Fig. 4. Expression of IFN-γ, IL-2 and IL-10 cytokines and viral *tax/rex* mRNA as detected by RT-PCR from day 0 to day 14 in HTLV-1-infected squirrel monkeys. Animals (A029C, A050C, A115C and 96070) were infected with an HTLV-1-transformed cell line, while the control animal (A118C) was inoculated with the same number of Jurkat cells. To compare cytokine expression in different samples, the ratio cytokine/GAPDH was calculated by measuring the density of each corresponding band. Values represent the mean cytokine expression of four animals. Errors bars correspond to S.E.M. among four animals.

activity (see review by (Katze et al., 2002). IFN-γ is an important regulator of innate and adaptive immune responses and is produced only by certain cells of the immune system, including NK cells, $CD4^+$ T helper 1 (Th1) cells and $CD8^+$ cytotoxic T cells. IFN-γ expression is commonly observed during early infection, but prevailing dogma considers that this expression has a suppressive effect on T-cell responses (Dalton et al., 2000; Refaeli et al., 2002). Nevertheless, recent works from Whitmire et al. (2005a, 2005b) have challenged this model. Indeed it was found that IFN-γ acts directly to increase the number both of $CD4^+$ and $CD8^+$ T-cells during acute viral infection (Whitmire et al., 2005a, 2005b). Based on our data, our hypothesis is that early expression of IFN-γ by NK and T-cells could facilitate T-cell expansion. The subsequent

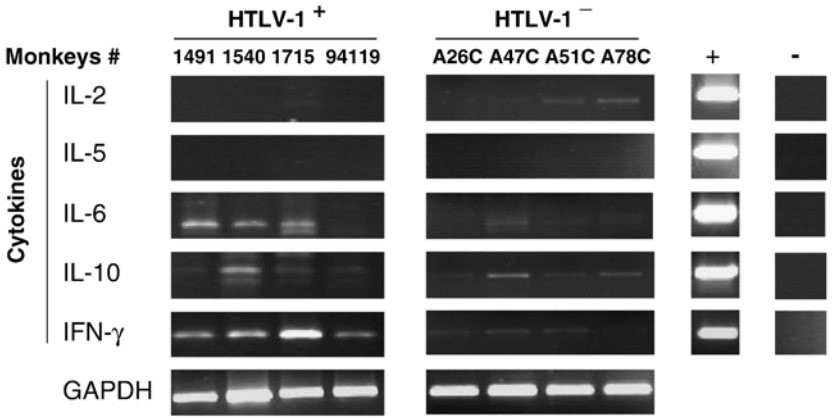

Fig. 5. RT-PCR pattern of Th1 and Th2 cytokine expression, including IL-2, IL-5, IL-6, IL-10 and IFN-γ, in fresh PBMCs (without culture) isolated from chronically HTLV-1-infected monkeys (animals 1491, 1540, 1715, 94119) or from uninfected control animals (26C, 47C, 51C, 78C). The positive control was a competitive plasmid containing specific primers for each cytokine; the negative control was the plasmid alone.

Table 1
Expression of IFN-γ, determined by quantitative RT-PCR, and HTLV-1 proviral load in PMBCs from monkeys chronically infected with HTLV-1

Monkey #	Ratio IFN-γ/G3PDH (×10^2)			Proviral load % of PBMCs[a]
	Total PBMCs	CD4$^+$ cells	CD8$^+$ cells	
1491	100	460	20	6.6
1540	50	22	60	0.9
1715	50	30	30	4.2
94119	5	30	<1	0.15
A26C[b]	<1	<1	<1	Undetectable

[a] Percentage of HTLV-1 infected cells in the PBMCs.
[b] Control, uninfected monkey.

expression of this cytokine on activated cells could lead to apoptosis and to the suppression of the immune response. This immunosuppressive effect of IFN-γ could therefore explain the expansion of infected cells over time and the occurrence of the disease. In our non-human primate model, we could not show which cellular subtype is responsible for IFN-γ production during primary HTLV-I infection. It is also possible that during early infection, infected cells expressed IFN-γ. Further experiments are needed to address this question.

Besides IFN-γ, IL-2 and IL-10 were also expressed transiently during the first week of infection. IL-2 is the major autocrine growth factor for T lymphocytes, and the quantity of IL-2 synthesized by activated CD4 cells is an important determinant of the magnitude of the T cell-dependent immune response (Horvat et al., 1991; Swain, 1991). IL-2 also stimulates the synthesis of other T cell-derived cytokines, such as IFN-γ. Similar to our observation in the squirrel monkey model, a high rate of expression of IL-2 has been reported during primary infection of macaques with SIV (Benveniste et al., 1996). IL-10, a regulatory cytokine is also essential for initiation of the humoral immune response and for activation of antigen-presenting cells. Treg cells (CD4$^+$ CD25$^+$) control the immune balance by secreting IL-10 (Vieira et al., 2004). The lack of antibodies that cross-react with CD4$^+$ CD25$^+$ from *Saimiri* monkeys obviates such an investigation.

In the present study we have also shown that *tax/rex* mRNA could be detected only shortly after infection but not thereafter. These results confirm our previous data on the *tax* expression during primary infection (Kazanji et al., 2000). We have previously demonstrated that *tax/rex* expression could be detected in the PBMCs, spleen and lymph nodes 6 days post infections of infected monkeys but was undetectable after 12–14 days. We have also shown that the expression of *tax/rex* mRNA during primary infection is not due to the inoculated cells but results from newly infected cells in the animals (Kazanji et al., 2000).

The profile of cytokine expression during latency indicates that two cytokines, IL-6 and IFN-γ are up-regulated in the PBMCs of chronically HTLV-1-infected monkeys and not in uninfected monkeys. IL-6 up-regulation appears to be related to the proviral load. IL-6 could play a role in the expansion of infected cells (i.e. proviral load) due to its anti-apoptotic effect on CD4+ cells (Ayroldi et al., 1998) and could reflect an inflammatory state. We showed previously that three monkeys (1491, 1715 and 1540) had expansion of HTLV-1-infected T cell clones (Debacq et al., 2005; Mortreux et al., 2001), consistent with the results of a recent study showing higher levels of IFN-γ in the sera of ATLL or HAM/TSP patients and asymptomatic

Table 2
Pools containing the overlapping peptides covering the entire Tax protein and used for in vitro stimulation of PBMCs from HTLV-I infected monkeys

Sequences of peptides used for in vitro stimulation	
Pool 1	
Tax 11–25	LLFGYPVYVFGDCVQ
Tax 21–35	GDCVQGDWCPISGGL
Tax 31–45	ISGGLCSARLHRHAL
Pool 2	
Tax 61–75	GRVIGSALQFLIPRL
Tax 71–85	LIPRLPSFPTQRTSK
Tax 81–95	QRTSKTLKVLTPPIT
Pool 3	
Tax 91–105	TPPITHTTPNIPPSF
Tax 101–115	IPPSFLQAMRKYSPF
Tax 111–125	KYSPFRNGYMEPTLG
Pool 4	
Tax 121–135	EPTLGQHLPTLSFPD
Tax 131–145	LSFPDPGLRPQNLYT
Tax 141–155	QNLYTLWGGSVVCMY
Pool 5	
Tax 151–165	VVCMYLYQLSPPITW
Tax 161–175	PPITWPLLPHVIFCH
Tax 171–185	VIFCHPGQLGAFLTN
Pool 6	
Tax 181–195	AFLTNVPYKRIEKLL
Tax 191–205	IEKLLYKISLTTGAL
Tax 211–225	DCLPTTLFQPVRAPV
Pool 7	
Tax 221–235	VRAPVTLTAWQNGLL
Tax 241–255	LTTPGLIWTFTDGTP
Tax 251–265	TDGTPMISGPCPKDG
Pool 8	
Tax 261–275	CPKDGQPSLVLQSSS
Tax 265–279	GQPSLVLQSSSFIFH
Tax 271–285	LQSSSFIFHKFQTKA
Pool 9	
Tax 281–295	FQTKAYHPSFLLSHG
Tax 291–305	LLSHGLIQYSSFHNL
Tax 301–315	SFHNLHLLFEEYTNI
Pool 10	
Tax 311–325	EYTNIPISLLFNEKE
Tax 315–329	IPISLLFNEKEADDN
Tax 321–335	FNEKEADDNDHEPQI
Pool 11	
Tax 331–345	HEPQISPGGLEPPSE
Tax 339–353	GLEPPSEKHFRETEV
Pool 12	
Irrelevant peptide[a]	GILGFVFTL

[a] Peptide located on the matrix protein of Influenza A virus.

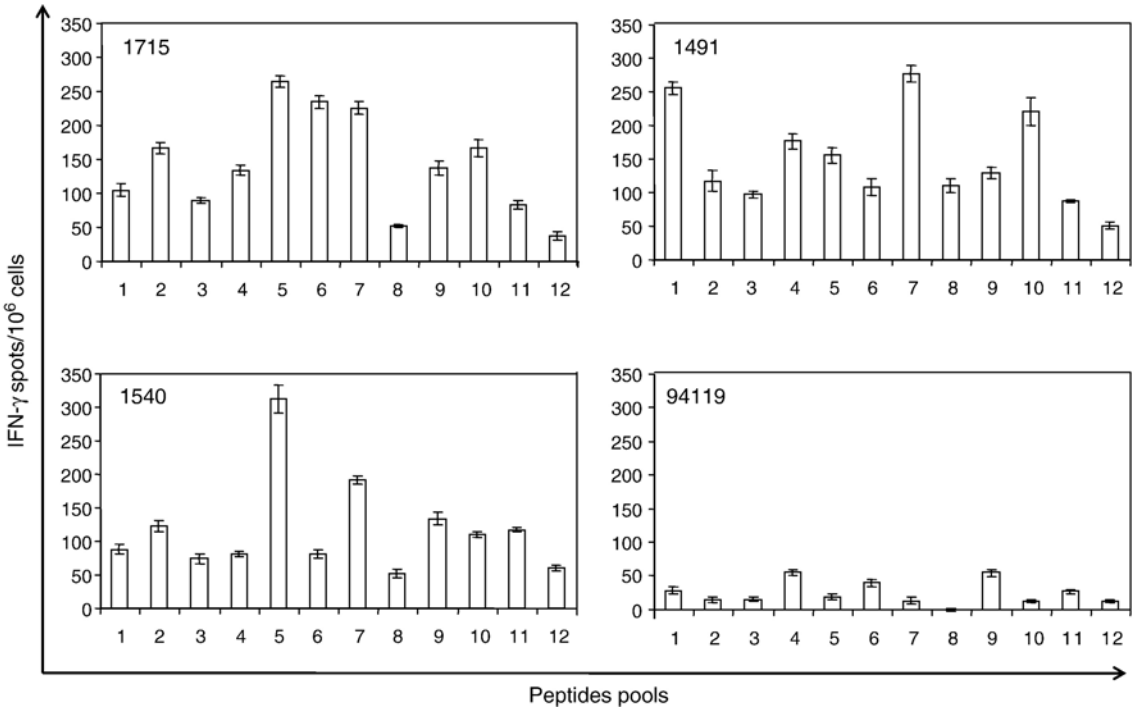

Fig. 6. IFN-γ expression in four chronically infected monkeys determined by the ELISPOT assay. Isolated fresh PBMCs from chronically infected monkeys were activated with 11 peptides pools as shown in Table 2 (each pool contains three overlapping Tax peptides). Pool 12 contained the irrelevant peptide. The numbers of spot-forming cells were calculated after subtraction of background spots from wells containing cells and medium only, with no peptide.

carriers than in healthy volunteers (Inagaki et al., 2006; Santos et al., 2004). Inagaki et al. (2006) also found a significantly higher level of IL-6 in ATLL patients but not in asymptomatic carriers. The difference from our data might be due to our model or linked to the proviral load, as Inagaki's study did not consider this variable. IFN-γ expression appeared to be associated with HTLV-1 infection, and our data suggest that IFN-γ is produced by monkey Th1 cells or NK cells as an immune response to HTLV-1-infected cells in the host. We previously demonstrated that animals 1491 and 94119 showed a $CD4^+$ lymphocytosis, with the presence of flower cells and the evidence of an expansion of a limited number of clones ($n<10$) (Debacq et al., 2005; Heraud et al., 2006). These features are similar to that observed in HTLV-1 infected human individuals suffering of chronic or smouldering ATL. The two animals with a chronic ATL are those in which the main IFN-γ-expressing cells were $CD4^+$ T-cells. IFN-γ trans-activation in HTLV-1-infected cells (Tax-dependent or -independent) might explain this phenomenon. In our model, it is still unclear whether the T cells that produce IFN-γ and/or IL-6 are infected cells or simply activated cells. Other studies are needed to explore this question. We did not observe significant expression of IL-2 during latency, consistent with the results of Inagaki et al. and Hanon et al. in vivo and in vitro (Hanon et al., 2001; Inagaki et al., 2006).

In a previous study, we showed the presence of a cytolytic T-cell response against Tax in enriched $CD8^+$ T cells from HTLV-1-infected squirrel monkeys. This response was detected 2 months after infection and remained detectable thereafter (Kazanji et al., 2000). We confirm these results in our new study by demonstrating that overlapping Tax peptides are able to stimulate the production of IFN-γ in fresh PBMCs from chronically infected monkeys. We also showed in our non-human primate model that the frequency of T cells secreting IFN-γ might be associated with the proviral load and with pathogenicity. We could not investigate which cells are involved in this mechanism; however, Kubota et al. (2000) found a positive correlation between a high HTLV-1 proviral load and an increased level of HTLV-1-specific $CD8^+$ lymphocytes in HTLV-1-infected individuals, particularly in HAM/TSP patients.

In conclusion, examination of cytokine expression during primary and chronic infection with HTLV-1 infection provides information about the dynamics of the immune response in our non-human primate model. Th1/Th2 regulatory cells, by expressing cytokines, play a critical role in controlling the immune balance that prevents severe diseases like ATLL. Our model of experimental infection is suitable for studying the relation between host and virus, and further studies are needed to determine whether the different patterns of cytokine and chemokine response during primary HTLV-1 infection are associated with the long latency by controlling HTLV-1 replication over time. Such studies would provide important information for the development of immune-based therapeutic strategies.

Materials and methods

Animals and HTLV-1 inoculation

Twelve 6-year-old male squirrel monkeys from the primate-breeding centre of the Pasteur Institute of French Guiana were

used. The animals were inoculated intravenously with 5×10^7 monkey HTLV-1-transformed cells, as described previously (Kazanji et al., 1997b). They were bled at various times after inoculation, and antibodies against HTLV-1 were measured by enzyme-linked immunosorbent assay (ELISA; Diagnostic Biotechnology Ltd, Singapore). HTLV-1 infection was confirmed by western blotting (HTLV blot 2.3, Diagnostic Biotechnology Ltd, Singapore).

Cloning, expression and generation of anti-squirrel monkey CD8$^+$ polyclonal antibodies

We have shown previously that mouse anti-human CD4 monoclonal antibodies recognize CD4$^+$ cells from squirrel monkeys, whereas mouse anti-human CD8 monoclonal antibodies do not (Kazanji, 2000). In an attempt to develop this anti-CD8 antibody, we cloned and sequenced squirrel monkey CD8 cDNA (Ureta-Vidal et al., 1999). 293T cells were transfected with the recombinant plasmid using the PolyFect® reagent (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The identity of CD8-α protein was studied by western blotting with an anti-His monoclonal antibody and an anti-β-tubulin antibody as controls, as described previously by Meertens et al. (2004). As a control, 293T cells were either mock-transfected or transfected with pcDNA4-LacZ control plasmid (Invitrogen Corporation, Carlsbad, CA, USA) (Fig. 1). To generate a mouse anti-squirrel monkey CD8 antibody, 4-week-old Balb/c mice (IFFA-CREDO, L'Arbesle, France) were given an intramuscular injection into the tibia anterior muscle of 50 μl of phosphate-buffered saline containing 400 μg of the naked DNA expression vector pcDNA3.1-His/CD8α,as previously described (Kazanji et al., 1997a). The mice were bled at various times after immunization, and sera were collected and screened by ELISA, as described previously (Kazanji et al., 2006), with a synthetic peptide (PTFLLYISQTKPKVA) located on the complementary determining region 2 (CDR2, aa 46–60) of the squirrel monkey CD8α chain (Ureta-Vidal et al., 1999). The specificity of the polyclonal antibody was also evaluated by flow cytometric analysis, as described previously (Kazanji et al., 2006). As shown in Fig. 1B, 49% of total PBMCs were CD8$^+$ cells (B1), and 76% of CD2$^+$ cells from CD4$^+$-depleted cells fraction were CD8$^+$ cells (B2).

Enzyme-linked immunospot (ELISPOT) assay for IFN-γ detection

The ELISPOT assay was carried out as described previously (Kazanji et al., 2006). Briefly, multiscreen filtration plates (MAIPS4510, Millipore, Bedford, MA, USA) were coated with 15 μg/ml anti-IFN-γ mAb (1-D1K, Mabtech, Sweden) overnight at 4 °C. The plates were then washed five times with RPMI-1640 medium and blocked with RPMI 1640 supplemented with L-glu, pen/strep and 10% fetal calf serum for 2 h. Then, 2×10^5 PBMCs in the above medium were added in a total volume of 200 μl in the presence or absence of 10 μg/ml of relevant peptide and incubated for 40 h at 37 °C. The cells were then incubated with 100 μl of 1 μg/ml biotinylated anti-IFN-γ mAb 7-B6-1 (Mabtech, Sweden) for 3 h at room temperature. After washing, the substrate BCIP/NBT (Kirkegaard and Perry Labs, Gaithersburg, MD, USA) was added, and the solution was incubated in the dark for 10–20 min until the appearance of dark-blue spots. The reaction was stopped by washing with cold tap-water, and the spots were counted with an Immunospot Image Analyzer (Zeiss, Oberkochen, Germany).

Cell isolation and flow cytometry analysis

PBMCs were separated from blood containing EDTA by sucrose density centrifugation with a Ficoll-Paque gradient (Sigma-Aldrich Co., St Louis, MO, USA) and were then washed three times with PBS. A CD4$^+$ T cell isolation kit and anti-mouse T cell lymphocyte kit microbeads (Miltenyi Biotec Ltd, Surrey, England) were used according to the manufacturer's recommendations to enrich or deplete the respective subpopulations. Lymphocyte subsets were determined by flow cytometric analysis with a FACScan (Becton Dickinson, Mountain View, CA, USA). Cells were washed with PBS containing 7% normal goat serum (Sigma-Aldrich Co., St Louis, MO, USA) and then incubated for 30 min at room temperature with various combinations of fluorescence (FITC)-conjugated monoclonal antibodies (all from Becton Dickinson). The stains used throughout the study were CD2-PE (RPA-2.10), CD4-FITC (Leu 3a) and CD16-FITC (3G8) monoclonal antibodies. For CD8 phenotyping assay, a polyclonal CD8 antibody produced in our laboratory associated with a secondary anti-mouse IgG FITC conjugate (Becton Dickinson) was used. Cells were run ungated. For analysis, lymphocyte gates were set with CellQuest™ software (Becton Dickinson). Lymphocyte subpopulations were calculated from the total white cell values obtained from a complete blood count performed with PENTRA 60 C+ (ABX Diagnostics, Montpellier, France).

Reverse transcription polymerase chain reaction (RT-PCR) and quantification of cytokine gene expression

In order to evaluate the expression of the different cytokines involved in the immune response to HTLV-1 infection, we used a previously described method (Heraud et al., 2002) based on competitive PCR and a competing plasmid, which has a cytokine sequence containing a GAPDH sequence. The cytokine and GAPDH primer-binding sites are identical to those on the sample cDNA, thus competing for amplification, although the intervening sequence is either longer or shorter. Co-amplification of competitor and sample cDNAs with one set of primers results in products of different sizes. Competitive PCR was performed in a GeneAmp® PCR System 9700 thermocycler (PE Biosystems, Foster, CA, USA) for 35 cycles, as previously described (Heraud et al., 2002). After amplification, the products of each reaction were separated on 2% TAE agarose gel, stained with ethidium bromide and photographed under 300 nm ultra-violet trans-illumination (Bioblock Scientific) with a CCD video camera module (BIO-PRINT® system

from Vilber Lourmat, Torcy, France). The digitalized image was analysed with BIO-1D software (Vilber Lourmat, Torcy, France). The fluorescence density of the target and competitor bands was calculated from the volume representing the sum of all the intensities of pixels making up the whole band. The results are expressed as cytokine:GAPDH mRNA ratio, as previously described (Pinelli et al., 1999; Rottman et al., 1996).

Detection of HTLV-1 proviral load

Genomic DNA was extracted from PBMCs by the method described by Ibrahim et al. (1994). The HTLV-1 proviral load was measured as previously described (Mortreux et al., 2001) by an accurate, reproducible, quantitative PCR method involving a dual-labelled fluorogenic probe (ABI PRISM 7700 Sequence detection system). The lower limit of detection is 10 copies per 1.5×10^5 PBMCs.

RT-PCR detection of tax/rex mRNA

Total cellular RNA was extracted from PBMCs and tissues with TRIzol reagent (Invitrogen, Frederick, MD, USA). The RNA was suspended in water and diluted to a final concentration of 0.1 μg/μl. It was then mixed with 15 pmol of the random hexamer primers in a final volume of 12.7 μl. RT and cDNA synthesis were performed as previously described (Kazanji et al., 2000). Immediately after cDNA synthesis, a semi-nested PCR was carried out on the *pX* region, with RPX3 and RPX5 as the outer primers and RPX3 and RPX4 as the inner primers (Kinoshita et al., 1989). To confirm the presence of amplifiable cDNA in the samples, the primers GAPD5ScF (sense) and GAPD3ScR (antisense) were used as described previously (Heraud et al., 2002).

Acknowledgments

We thank J.F. Pouliquen, Julie Vandekerkhove and E. Francisot for technical help. We are grateful to Dr. A. Gessain for critical reading of the manuscript. We also thank the Ministry of Research (Programme de Recherche fondamentale en Microbiologie des Maladies Infectieuses et Parasitaires), the Association pour la Recherche contre le Cancer (grants Nos. 7590 and 5700 to MK) and the Virus Cancer Prevention association for financial support. J.-M. Héraud was a recipient of a fellowship from the Caisse Nationale d'Assurance Maladie et Maternité des Travailleurs Non Salariés des Professions Non Agricoles, La Fondation pour la Recherche Médicale, La Région Guyane and the Pasteur Institute Network fellowship programme. The experiments performed comply with French laws relative to animal experimentation.

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